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Effects of histone deacetylase inhibitors on regenerative cell responses in human dental pulp cells

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Abstract

Aim To investigate the growth, migratory and adhesive effects of Trichostatin A (TSA) and Valproic acid (VPA), two HDACis, on hDPSCs.

Methodology To verify that TSA or VPA functions as an HDAC inhibitor, the expressions of histone H3 and H4 were examined using Western blotting analysis. hDPSC growth and metabolic activity was evaluated by MTT viability analysis at different time-points and by cell count experiments. The expression of cell cycle regulatory and the apoptosis associated proteins were examined by Western blot analysis. Migration effects were investigated using wound healing and transwell migration assays. An adhesion assay was also performed in the presence and absence of HDACis. The levels of chemokines and adhesion molecules relevant to repair in hDPSCs were also assessed by qRT-PCR and western blot analysis. The data were analyzed, where appropriate, using the Student's t-test or one-way ANOVA followed by the Student-Neumann-Keuls test using SPSS software.

Results TSA and VPA enhanced acetylation of histone H3 and H4 ($P < 0.05$). Significant increases ($P < 0.05$) in MTT levels in hDPSCs were observed after treatment with TSA (2 nmol/L and 20 nmol/L) or VPA (1 mmol/L and 10 mmol/L). Cell numbers were not significantly affected at the concentration of TSA (0.2 nmol/L -10nmol/L) or VPA (0.01 mmol/L-100 mmol/L) applied compared with the control at 3, 5 or 7 days ($P>0.05$). At the same time, the expression of Cdx2, cyclinA was up-regulated by 2 nmol/L TSA and 1 mmol/L VPA ($P < 0.05$). Higher TSA or VPA concentrations induced apoptosis in hDPSCs in the cell count and apoptosis experiments ($P < 0.05$). Moreover, TSA and VPA significantly depressed the expression of Cdx2, cyclinA ($P < 0.05$), while it significantly improved the level of p21 ($P < 0.05$). TSA and VPA promoted migration and adhesion of hDPSCs ($P < 0.05$). The levels of chemokines and adhesion molecules were significantly up-regulated after exposure of hDPSCs to 20 nmol/L TSA or 1 mmol/L VPA ($P < 0.05$).

Conclusions HDACis at specific concentrations promoted proliferation, migration and adhesion of hDPSCs, which may contribute to novel regenerative therapies for pulpal disease treatment.

Introduction

Pulpal healing after direct pulp exposure involves a complex interplay of molecular and cellular processes. The sequence of events in pulpal healing is similar to those after injury of other connective tissues (Schröder 1985). Schröder described the first stage as involving vascular and inflammatory cell growth, migration and adhesion to control and eliminate the injurious challenge. Subsequently, repair occurs with cell growth and metabolic activity, migration and adhesion of relevant cell types. Notably regeneration of the dentine-pulp complex after severe injury involves differentiation of stem/progenitor cells, including human dental pulp stem cells (hDPSCs), to provide a new generation of odontoblast-like cells which replace dying primary odontoblasts (Schröder 1985). hDPSCs represent a population of multipotent progenitor cells, which have been reported to show high proliferative, migratory and adhesive potential and are important for dental tissue regeneration (Gronthos *et al*. 2011). The identification of regulatory factors which influence the cell growth, migration and adhesion of hDPSCs will be important in the development of new therapeutic strategies to treat dental tissue injury.

Cell proliferation, migration and adhesion are important for homeostatic tissue maintenance and the tissue regeneration. Some cell cycle regulatory proteins, chemokines and adhesion molecules were reported to be involved to mediate proliferation, migration and adhesion process. p21 overexpression seems to inhibit growth and/or promote cell differentiation, results in cell cycle arrest in G1(Xiong *et al*. 1993). Cyclin A is a member of the cyclin family, a group of proteins that function by regulating progression through the cell cycle (Soucek *et al*. 1997). The binding between Cdk2 and cyclin A is required to progress through the S phase (Morgan 1995). It has been demonstrated SDF-1/CXCR4 axis were required for mobilization and recruitment of BMSCs (Peled *et al*. 1999, Herberg *et al*. 2013). MCP-1 is also considered as a migratory stimulus (Schwarz *et al*. 2004). Fibroblast growth factor-2 (FGF2) was proved to enhance the proliferation and migration potential of oligodendrocyte progenitor cells (OPCs) and inflamed pulp tissue of human functional primary teeth (iSHFD)(Kim *et al*. 2014, Azin *et al*. 2015). In

addition, it has been reported that cell adhesive activity is dependent on FN type III domain fragments (Yun *et al*. 2015). The intercellular adhesion molecule-1(ICAM-1) can mediate the adherence process between mesenchymal stem cells (MSC) and endothelial progenitor cells (EPC) (Guo *et al*. 2016). The expression of VCAM-1 was enhanced via PDGFRβ and the activation of Src in a ligand-independent manner during the cell-cell adhesion process in MSCs (Aomatsu *et al*. 2014). Specific beta1 integrins mediate adhesion, migration, and differentiation of neural progenitors derived from the embryonic striatum (Tate *et al*. 2004). These molecules may regulate wound healing-related responses within the dentine-pulp complex via mediating proliferation, migration and adhesion process.

Acetylation of DNA-associated histone and non-histone proteins alters gene expression and induces a range of cellular effects, which are enzymatically balanced by two groups of nuclear enzymes histone acetyltransferases (HATs) and histone deacetylases (HDACs). HAT activity relaxes the architecture of human chromatin increasing transcription, while HDAC activity condenses chromatin structure leading to gene silencing (Richon *et al.* 2002). There are 18 identified human HDACs, 11 of which are zinc-dependent, separated into 4 classes (Su *et al*. 2008) Although HDACs possess similar enzymatic activities, loss of function experiments have attributed highly specific roles to individual HDAC proteins in development and differentiation (Haberland *et al.* 2009). Differences in tissue expression are also evident with class I members ubiquitously expressed in the cell, while class II enzymes are tissue-restricted in their expression (Montgomery *et al*. 2007).

Histone deacetylase inhibitors (HDACis) are natural or synthetic epigenetic modifying molecules that alter the homeostatic enzyme balance increasing the level of protein acetylation and inducing pleiotropic effects in cell growth, differentiation, angiogenesis, and inflammation (Hochedlinger & Plath 2009, Duncan *et al*. 2011, Williams *et al*. 2011). The HDACi Trichostatin A (TSA), Valproic acid (VPA) and Suberoylanilide hydroxamic acid (SAHA) are commonly used pan-inhibitors being active against all zinc dependent HDAC classes (I, II, IV), however, the

use of selective inhibitors may also become a focus of future work once the roles of individual HDACs are elucidated (Khan *et al*. 2008). Several pan-inhibitors have been demonstrated to promote differentiation and increase mineralization dose-dependently in primary dental pulp cell populations at relatively low concentrations (Duncan *et al*. 2013, Jin *et al*. 2013, Paino *et al*. 2014). Conversely, at selected low concentrations, HDACi have also been shown to stimulate expansion of stem cell populations (Liang *et al*. 2010, Mahmud *et al.* 2014).

The purpose of this study was to investigate the effects of relatively low concentrations of TSA and VPA on the responses of hDPSCs in terms of growth, migration, adhesion and the potential involvement of different chemokines and adhesion molecules. The null hypothesis is that TSA and VPA have no effects to the growth, migration and adhesion of hDPSCs in vitro.

Materials and Methods

Reagents

Trichostatin A(TSA) (7-[4-(Dimethylamino)phenyl]-N-hydroxy-4,6-dimethyl-7-oxo-2,4-heptadienamide) was purchased from InvivoGen (San Diego, CA). Valproic acid (VPA), 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) and dimethyl sulphoxide (DMSO) were obtained from Sigma (St Louis, MO, USA). Transwell chambers were purchased from Corning (Corning, N.Y, USA).

Cell culture

All experiments were performed with the approval of the Ethics Committee of the Fourth Military Medical University (FMMU), Xi'an, PR China. Freshly extracted teeth were collected from three human adults (18 to 22-year-old) with informed consent. Dental pulps were isolated and dissected into small pieces prior to digestion in a solution of 3 mg/mL type I collagenase with 4 mg/mL dispase (Sigma) for 45-60 min at 37°C. Subsequently, the digest was filtered through a 70 µm cell strainer (Becton/Dickinson, Franklin Lakes, NJ, USA). The single cell suspensions were

seeded in 35 mm culture dishes and maintained in culture medium consisting of α-MEM supplemented with 20% (v/v) foetal bovine serum (FBS; Hyclone), 2 mmol/L glutamine (Invitrogen), 100 units/mL penicillin G, 100 mg/mL streptomycin and 50 mg/mL ascorbic acid (Sigma) in a humidified atmosphere containing 5% CO_2 at 37°C. The medium was changed every 3 days. hDPSCs were characterized by flow cytometry and analysis of multiple lineage differentiation potential as described previously (He *et al*. 2013, Zhang *et al*. 2013). Cells between passages 2 to 5 were used in all experimentation.

Evaluation of cell number and cell viability

hDPSCs were seeded in 6-well plates at a density of 6×10^4 cells per well in 2mL α-MEM medium containing 10% (v/v) FBS. After 24 hours, TSA (0.2 nmol/L, 2 nmol/L, 20 nmol/L, 100 nmol/L, 500 nmol/L) and VPA (0.01 mmol/L, 0.1 mmol/L, 1 mmol/L, 10 mmol/L, 100 mmol/L) were supplemented in cultures and incubated for a further 1d, 3d, 5d or 7d. The positive control samples contained cells cultured in α-MEM not supplemented with TSA or VPA, whereas the negative control samples contained no cells. Cells were detached with Hank's balanced salt solution containing 2.5 g/L trypsin and 0.2 g/L EDTA and stained with 0.4% trypan blue before cell counting. Total number of cells/well was determined using a hemacytometer (Hausser Scientific, Horsham, PA, USA). These experiments were performed in triplicate on three separate experiments.

MTT assay

For the MTT assay, hDPSCs were seeded in 96-well plates at a density of 3×10^3 cells per well in 100μL α-MEM medium containing 10% (v/v) FBS and allowed to adhere for 24 hours. Samples were incubated for 24 hours in medium without FBS prior to treatment with 2% α-MEM medium containing a range of concentrations of TSA (0.2 nmol/L - 500 nmol/L) and VPA (0.01 mmol/L - 100 mmol/L). Control cells were cultured in 5% α-MEM medium without TSA or VPA. Each group contained 5 wells, and the medium was changed every 3 days. Cell metabolic activity was estimated from MTT data on days 1, 3, 5 and 7 respectively. Briefly, on the day of harvest, 20μL

MTT (5 mg/mL in PBS) was added to each well and incubated at 37°C for 4 hours. The culture medium was then replaced with 150µL DMSO per well and plates were agitated at room temperature for 10 min. Cell growth and viability were determined from the absorbance of the converted dye at a wavelength of 570 nm on a multi-plate reader (BIO-TEK, Winooski, VT, USA).

Wound healing assay

Approximately 3×10^5 hDPSCs were seeded into 100 mm culture dishes without type-1 collagen coated and grown to confluence. Cells were incubated for 24 hours in medium without FBS prior to a scratch wound being made through the confluent layer of cells using a 200µL pipette tip. After wounding, cells were washed with phosphate buffered saline (PBS) to remove cell debris and then incubated with 2% α-MEM medium and HDACis (TSA 2 nmol/L, TSA 20 nmol/L, VPA 1 mmol/L and VPA 10 mmol/L) for 24 hours. Photomicrographs of the scratch were taken at 0 and 24 hours post-wounding using a phase-contrast microscope (Olympus, Tokyo, Japan). Scratch-wound closure rate was determined using i-Solution software (iMTechnology, Korea), and the shortest distance between cells that had migrated into the wounded area and their respective starting points was determined. The numbers of cells that had migrated to the scratched area after 24 hours were counted and wound closure was quantified.

Transwell Migration Assay

Cell migration was also evaluated using a two-chamber Transwell system (8 µm pore size and 6.5 mm diameter). Briefly, 5×10^4 hDPSCs were suspended in 100µL serum free α-MEM and seeded in the top chamber of the Transwell, and 700µL of serum-free α-MEM in the presence or absence of HDACis (TSA 2 nmol/L, TSA 20 nmol/L, VPA 1 mmol/L and VPA 10 mmol/L) were added to the lower chamber. After 24 hours incubation at 37°C in 5% CO_2 in air, cells remaining on the top surface of the filter were removed using a cotton swab and the hDPSCs that had traversed the membrane to the bottom chamber were fixed for 10 min with 4% paraformaldehyde and stained with haematoxylin for 30 min. To quantify the migrated cells, ten random microscopic fields per

filter at 200X magnification were selected for cell counting by a phase-contrast microscope (Olympus, Tokyo, Japan). Measurements were performed in triplicate and results calculated as means for each experiment.

Adhesion Assay

For the cell adhesion assay, 96-well plates were coated with type I collagen (40 mg/L in PBS) overnight at 4°C. The wells were then blocked for 2 hours at 4°C with 100µL 1% (w/v) BSA in PBS. Where indicated, HDACis (TSA 2 nmol/L, TSA 20 nmol/L, VPA 1 mmol/L and VPA 10 mmol/L) were combined with the cells prior to plating. Cells were trypsinized, resuspended in serum-free medium at a cell density of 1×10^5 cells/mL and 100µL of the cell suspension were added to each well. Cells were incubated for 1 hour at 37°C. Non-adherent cells were removed by washing with PBS, and adherent cells were fixed in 4% formaldehyde for 20 min. The cells were then stained with 0.1% cresyl violet acetate for 30 min followed by a thorough wash with water, and the stain solubilised with 10% acetic acid. The solubilised stain was quantified at 595 nm using a multiplate reader (BIO-TEK, Winooski, VT, USA). Experiments were performed in triplicate and results calculated as means for each experiment.

Quantitative Real-time Reverse-transcriptase Polymerase Chain Reaction (qRT-PCR)

After exposure to HDACis (TSA 20 nmol/L or VPA 1 mmol/L) for the indicated time period, total RNA was extracted from cells using Trizol reagent (Life Technologies Inc.) and this was DNase I (RNase-free, RQ1; Promega, Madison, WI, USA) digested. One microgram of total RNA was used as a template for synthesis of first-strand complementary DNA using oligo-dT priming and an Omniscript RT kit (Qiagen, Inc., Valencia, CA, USA). qRT-PCR analyses were performed using an ABI Prism 7500 Real-Time PCR System (Applied Biosystems, Foster City, CA, USA) with SYBR Green PCR master mix reagent (Takara, Otsu, Japan) for 35-cycles. The denaturing, annealing, and extension conditions for each PCR cycle were 95°C for 10 s, 60°C for 15 s, and 72°C for 10 s, respectively. The relative amount or fold-change of the target gene was normalized relative-to the level of human glyceraldehyde-3-phosphate dehydrogenase （GAPDH）

and the control (untreated cells). Primer sequences (Sangon Biotech (Shanghai) Co., Ltd.) for GAPDH, along with the chemokines - CXC chemokine receptor type 4 (CXCR4)，fibroblast growth factor 2 (FGF2), monocyte chemoattractant protein 1 (MCP-1), stromal cell-derived factor-1 (SDF-1) and the adhesion molecules - fibronectin (FN), intercellular adhesion molecule 1 (ICAM-1), vascular cell adhesion molecule 1 (VCAM-1) and beta1 integrin (Integrinβ1) are provided in Table 1.

Western blotting

Protein was extracted from $4x10^5$ DPSCs following seeding in a 60-mm dish, serum-starvation overnight, and treatment with HDACis for the indicated times. Samples containing 25–50 μg of total protein lysates (after Bradford estimation) were separated by SDS-PAGE and blotted onto a PVDF membrane (Millipore). All antibodies against the following proteins were used and purchased from Cell Signaling Technology (Beverly, MA, USA): acetylated histone H3, acetylated histone H4, cyclinA, p21 and Cdk 2, cleaved caspase-3 and -9, chemokines (CXCR4, FGF2, MCP-1 and SDF-1) and adhesion molecules (FN, ICAM-1, VCAM-1 and Integrin β1) and β-actin were applied in TBS-T buffer. The cells were washed 3 times in cold Tris-buffered saline and protein was collected in protein lysis buffer. Western blots were performed as previously described (He *et al*. 2012b). Finally, protein bands were visualized using an enhanced chemiluminescence Accepted Article system (Amersham, Piscataway, NJ, USA).

Statistical analysis

The data are expressed as means ± standard deviation from three independent experiments performed in triplicate. The data were analyzed, where appropriate, using the Student's t-test or one-way ANOVA followed by the Student-Neumann-Keuls test using SPSS software (version 17.0; SPSS, Chicago, IL, USA). A level of statistically significant difference was accepted at $P < 0.05$.

Results

Effects of HDACis on the growth and metabolic activity of hDPSCs.

In order to verify that TSA and VPA functions as an HDAC inhibitor, DPSCs were treated with TSA and VPA, and the expressions of histone H3、H4 were examined using Western blotting analysis. The expression of histone H3、H4 acetylation was increased in DPSCs (Fig.1A&B). It confirmed that HDAC inhibitors were working appropriately in the study. To examine the effects of HDACis (TSA or VPA) on hDPSC growth *in vitro* cells were cultured with TSA (0.2 nmol/L-500 nmol/L) or VPA (0.01 mmol/L-100 mmol/L) for 1, 3, 5 and 7 days, respectively and the effects assessed using the MTT assay. The results concealed that HDACis significantly increased at specific TSA (2 nmol/L and 20 nmol/L) or VPA (1 mmol/L and 10 mmol/L) concentrations, although cell growth activity were significantly decreased at higher concentrations and were similar to controls at lower concentration exposures (Fig.1C&D). Cell numbers were not significantly affected at the concentration of TSA (0.2 nmol/L-10 nmol/L) or VPA (0.01 mmol/L-100 mmol/L) applied compared with the control at 3, 5 or 7 days. While, growth of DPSCs was slight inhibited by 500 nmol/L TSA and 100 mmol/L VPA at 3, 5, 7 days (Fig.1E&F). In addition, the expression of cell cycle regulatory proteins Cdx2, cyclinA was up-regulation by 2 nmol/L TSA and 1 mmol/L VPA, but it was decreased by higher TSA or VPA. The expression of p21 was markedly increased in response to higher TSA or VPA (500 nmol/L TSA and 100 mmol/L VPA) treatment, but it was not significantly influenced by 2 nmol/L TSA and 1 mmol/L VPA. Meanwhile, the apoptosis experiment was used to determine whether higher TSA or VPA concentrations could induce apoptosis in hDPSCs. In fact, higher TSA or VPA (500 nmol/L TSA and 100 mmol/L VPA) induced cleavage of caspase-9 and -3 in DPSCs, which indicated that it had a growth inhibitory effect greater than TSA or VPA (Fig.2A&B).

Effects of HDACis on hDPSC migration and adhesion and related molecular expression.

HDACis stimulated the migration of hDPSCs *in vitro* (Fig. 3). Both TSA (2 nmol/L and 20

nmol/L) and VPA (1 mmol/L and 10 mmol/L) exposure significantly increased cell migration in a wound healing assay ($P < 0.05$) (Fig. 3A&C). Similar concentrations of TSA (2 nmol/L and 20 nmol/L) or VPA (1 mmol/L and 10 mmol/L) also stimulated the migration of hDPSCs in a Transwell assay with the greater effects observed with 20 nmol/L TSA or 1 mmol/L VPA ($P < 0.05$) (Fig.3B&D).Both TSA (2 nmol/L and 20 nmol/L) and VPA (1 mmol/L and 10 mmol/L) showed positive effects on the adhesion of hDPSCs after 1 hour treatment ($P < 0.05$) (Fig. 4A&B).

Furthermore, after exposure of hDPSCs to HDACis (TSA or VPA) for 24 hours, TSA (20 nmol/L) or VPA (1 mmol/L) gave rise to significant up-regulation of the expression of chemokines (CXCR4, FGF2, MCP-1 and SDF-1) and adhesion molecules (FN, ICAM-1, VCAM-1 and Integrin β1) ($P < 0.05$) (Fig. 4C&D). The western blot results also confirmed that TSA (20 nmol/L) or VPA (1 mmol/L) increased the expression of chemokines molecules and adhesion molecules ($P < 0.05$) (Fig. 2C-E).

Discussion

TSA and VPA are both potent pan-HDACis that suppress the activities of all eleven identified human class I, II and IV HDACs (Vanhaecke *et al.* 2004). It has been demonstrated that TSA at relatively low concentration (up to 50 nmol/L), increased the growth of hDPSCs, while 100 nmol/L TSA reduced growth rates with cell cytotoxicity (Jin *et al.* 2013). Interestingly, other primary dental pulp cell studies have demonstrated that TSA and VPA at low concentrations did not significantly promote or inhibit growth, but at higher concentrations were anti-proliferative (Duncan *et al*. 2013, Paino *et al*. 2014), supporting the conclusions of several other studies in which HDACi are described as anti-proliferative and pro-differentiation drugs (Xu *et al*. 2007, Marks 2010, Duncan *et al*. 2012). These apparently contradictory results indicate that in certain cell environments and concentrations of HDACi may play pivotal roles in regulating cell growth. This offers potential in selected environments for expansion or differentiation of hDPSC

populations for dentine-pulp complex repair. In the present study a similar trend was observed with TSA or VPA at 5 different concentrations. TSA (2 nmol/L and 20 nmol/L) or VPA (1 mmol/L and 10 mmol/L) demonstrated an increase in cell numbers for hDPSCs, while following treatment with TSA 20 nmol/L or VPA 1 mmol/L the greatest effects on hDPSCs were observed, although cell growth activity were significantly decreased at higher concentrations and were similar to controls at lower concentration exposures.

Interestingly, at the higher concentrations of HDACis applied significantly decreased cell numbers were detected implying an anti-proliferative effect as reported in other studies (Duncan *et al*. 2013, Paino *et al*. 2014). Cyclin A works from the late G1 phase through the M phase of the cell cycle, and forms a complex with cdk2 in the late G1-S phase (Sherr *et al.* 1994). cyclin A was reported that it may promote proliferative activity of endometrial adenocarcinoma (Kyushima *et al.* 2002). p21 overexpression seems to inhibit growth and/or promote cell differentiation, results in cell cycle arrest in G1(Xiong *et al*. 1993). In this study, low concentrations of TSA and VPA treatment promoted the expression of Cyclin A and cdk2. Higher TSA or VPA depressed the expression of Cyclin A and cdk2. However, G1 cell cycle arrest was induced, and the expressions of p21 were increased. In addition, higher TSA or VPA (500 nmol/L TSA and 100 mmol/L VPA) induced cleavage of caspase-9 and -3 in DPSCs, and it also slightly inhibited the growth of DPSCs in the cell count experiment. It indicated that low concentrations of TSA and VPA promoted the proliferation of hDPSCs, yet it had growth inhibitory effects at higher TSA or VPA.

Cell migration and adhesion is necessary for homeostatic tissue maintenance and the regeneration of injured organs and tissues (Gilbert 2003). Indeed, cells are needed to migrate and adhere to sites where they are needed (Singer & Clark 1999). hDPSCs are understood to give rise to odontoblast-like cells following tooth injury provided a conducive environment is present (Singer & Clark 1999, Alliot-Licht *et al.* 2005). Notably, hDPSCs grow，migrate, differentiate and adhere to enable replacement of the injured original odontoblasts, and subsequently they

synthesize and secrete tertiary dentine, while clinically results in the formation of a dentine bridge (Qvist 1975, Kitasako *et al*. 2008). It has been reported previously that HDACis SAHA and sodium butyrate (SB) inhibited the growth, migration and adhesion of human osteosarcoma cells (Zhang *et al*. 2013c). HDACi TSA enhanced proliferation, migration and adhesion of vascular smooth muscle cells by down-regulating thioredoxin 1 (Song *et al*. 2010). In the present study, TSA or VPA at certain concentrations significantly increased migration and adhesion of hDPSCs. Subsequent transcript analysis also indicated the potential involvement, of several chemokines and adhesion molecules.

Studies have demonstrated that neural stem cell proliferation and migration is regulated via the SDF-1α/CXCR4 pathway (Luo *et al.* 2014). Furthermore, CXCR4-overexpression in bone marrow mesenchymal stem cells (BMSCs) demonstrated enhanced migration in skin wounds in a SDF-1-expression-dependent manner, subsequently reducing healing times (Yang *et al.* 2013). The growth factors FGF2 and VEGF have also been shown to promote the proliferation, migration and adhesion of human periodontal ligament stem cells *in vitro* (Zhang *et al.* 2013b). Ox-LDL has also been shown to increase the migration and adhesive behavior of BMSCs via up-regulation of MCP-1 expression (Zhang *et al.* 2013a). Studies have also demonstrated that the mRNA expression of CXCR4, FGF2, MCP-1 and SDF-1 are up-regulated in response to HDACis (TSA 20 nmol/L or VPA 1 mmol/L) in hDPSCs and subsequently, the western blot results also confirmed that TSA (20 nmol/L) or VPA (1 mmol/L) increased the expression of chemokines molecules. These molecules may regulate wound healing-related responses within the dentine-pulp complex.

Previously, a fibronectin-calcium phosphate composite layer on hydroxyapatite has been shown to enhance stem cell adhesion, spreading and osteogenic differentiation *in vitro* and it is known that specific beta1 integrins mediate these processes (Tate *et al.* 2004, Sogo *et al.* 2007). Furthermore, NF-κB-induced up-regulation of adhesion molecules, such as ICAM-1, VCAM-1 and E-selectin, are reportedly key to many cell migratory regulated events (Koh *et al*. 2011,

Dieterich *et al*. 2013). The present data further support the potential involvement of these molecules in hDPSC motility and highlight that their transcript levels can be regulated by selected HDACi. This result was also confirmed through western blotting. Future analysis of HDACi upstream regulatory pathways in hDPSCs, such as NF-κB, is warranted as links have been demonstrated between HDACi-induced anti-inflammatory effects and HDACi (Gupta *et al*. 2010).

Conclusion

The HDACis (TSA or VPA) applied at specific concentrations *in vitro* promote a wound repair-related process in hDPSCs. These data further support the therapeutic potential of HDACis for use in dentine-pulp complex regeneration.

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Conflict of Interest statement

The authors have stated explicitly that there are no conflicts of interest in connection with this article.

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Figure Legend

Figure 1 Protein expression of acetylated histone H3 and H4 as measured by Western blot analysis (A&B). The effects of HDACis [C) TSA or D) VPA] on MTT levels in hDPSCs. The effects of TSA (E) or VPA (F) on cell number in hDPSCs. Data are the mean±S.D. of three independent experiments. *P < 0.05 represents a significant change compared with the control.

Figure 2 Cell apoptosis- and cycle-related protein expression in hDPSCs, as measured by Western blot analysis (A&B). The levels of chemokines and adhesion molecules were examined by Western blot analysis (C-D). Data are the mean±S.D. of three independent experiments. *P < 0.05 represents a significant change compared with the control.

Figure 3 A) Photomicrographs of the scratch were taken at 0 and 24 hours post wounding. C) Quantitative data from A) showing the number of migrated of cells. B) Cell migration assays were applied using a two-chamber Transwell system. D) Quantitative data from B) showing cell migration in response to HDACis. Data are the mean±S.D. of three independent experiments. *P < 0.05 represents a significant difference compared with the control. Scale bars indicate 100 µm.

Figure 4 The effects of HDACis on the adhesion of hDPSCs. Cells were incubated in HDACis. A) Adherent cells were fixed and stained and B) quantification was performed. The mRNA expression of chemokines (C) and adhesion(D) molecules examined in hDPSCs. Results are expressed as the mean±standard deviation of 3 independent experiments. *P < 0.05 represents a significant difference compared with the control. Scale bars indicate 100 µm.

Table 1 - Primer sequences used for qRT-PCR analysis

Gene	Code	Forword primer sequences	Reverse primer sequences
CXCR4	XM_005573045.1	TACACCGAGGAAATGGGCTCA	AGATGATGGAGTAGATGGTGGG
FGF2	XM_003816433.1	CGTGCTATGAAGGAAGATGGA	TGCCCAGTTCGTTTCAGT
MCP-1	XM_003996556.1	CCAAAGAAGCTGTGATCTTCAA	TGGAATC CTGAACCCACTTC
SDF-1	XM_005859482.1	GTCAGCCTGAGCTACAGATGC	CACTTTAGCTTCGGGTCAATG
FN	XM_006245159.1	TCCTTGCTGGTATCATGGCAG	AGACCCAGGCTTCTCATACTTGA
ICAM-1	NM_012967.1	ATCCATCCCACAGAAGCCTTCCTGC	GCCCACCTCCAGGAGGTCAGGGGTGT
VCAM-1	NM_001199834.1	CATGACCTGTTCCAGCGAGG	CATTCACGAGGCCACCACTC
Integrinβ	XM_0055701221.1	TGCAGTTTGTGGATCACTGATTG	CCTGTGGACTGTCGAGGCATAAG
GAPDH	XM_005569913.1	CCTGCACCACCAACTGCTTA	GGCCATCCACAGTCTTCTGAG

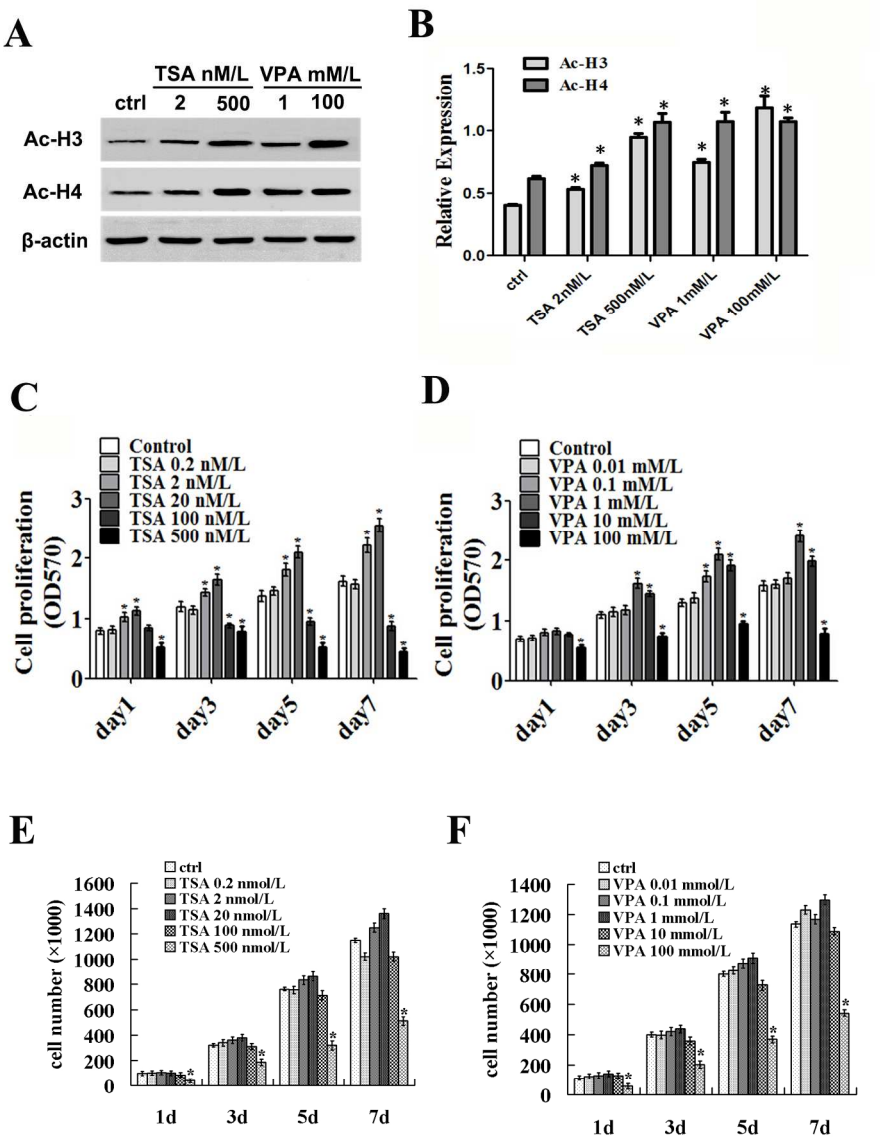
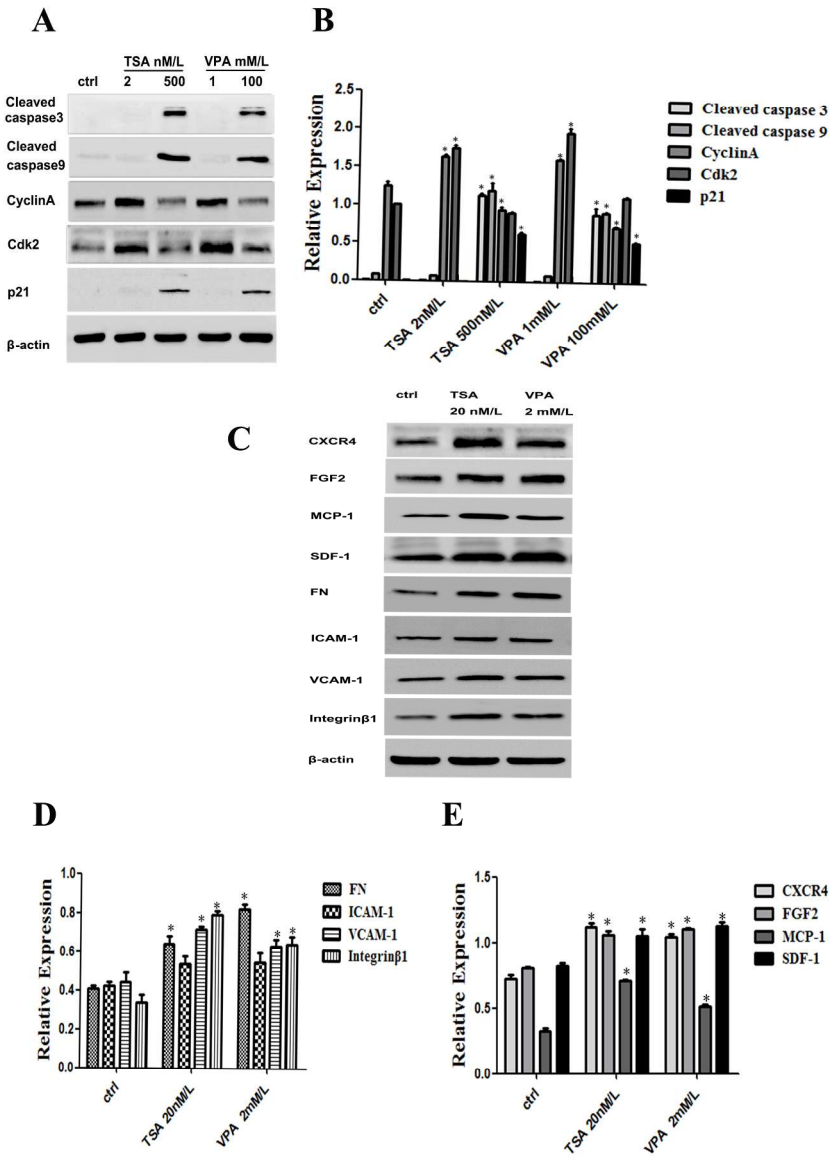


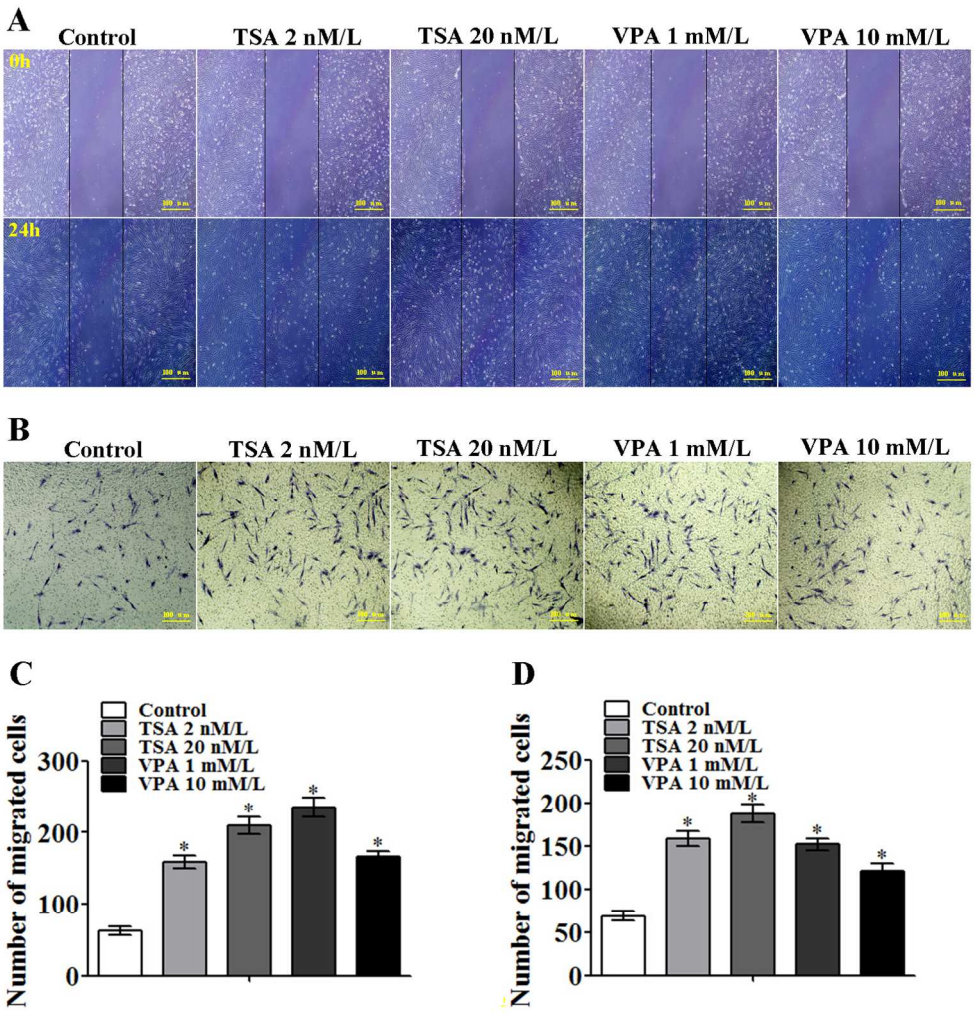
Figure 1. Protein expression of acetylated histone H3 and H4 as measured by Western blot analysis (A&B). The effects of HDACis [C) TSA or D) VPA] on MTT levels in hDPSCs. The effects of TSA (E) or VPA (F) on cell number in hDPSCs. Data are the mean±S.D. of three independent experiments. *P < 0.05 represents a significant change compared with the control.

194x247mm (300 x 300 DPI)



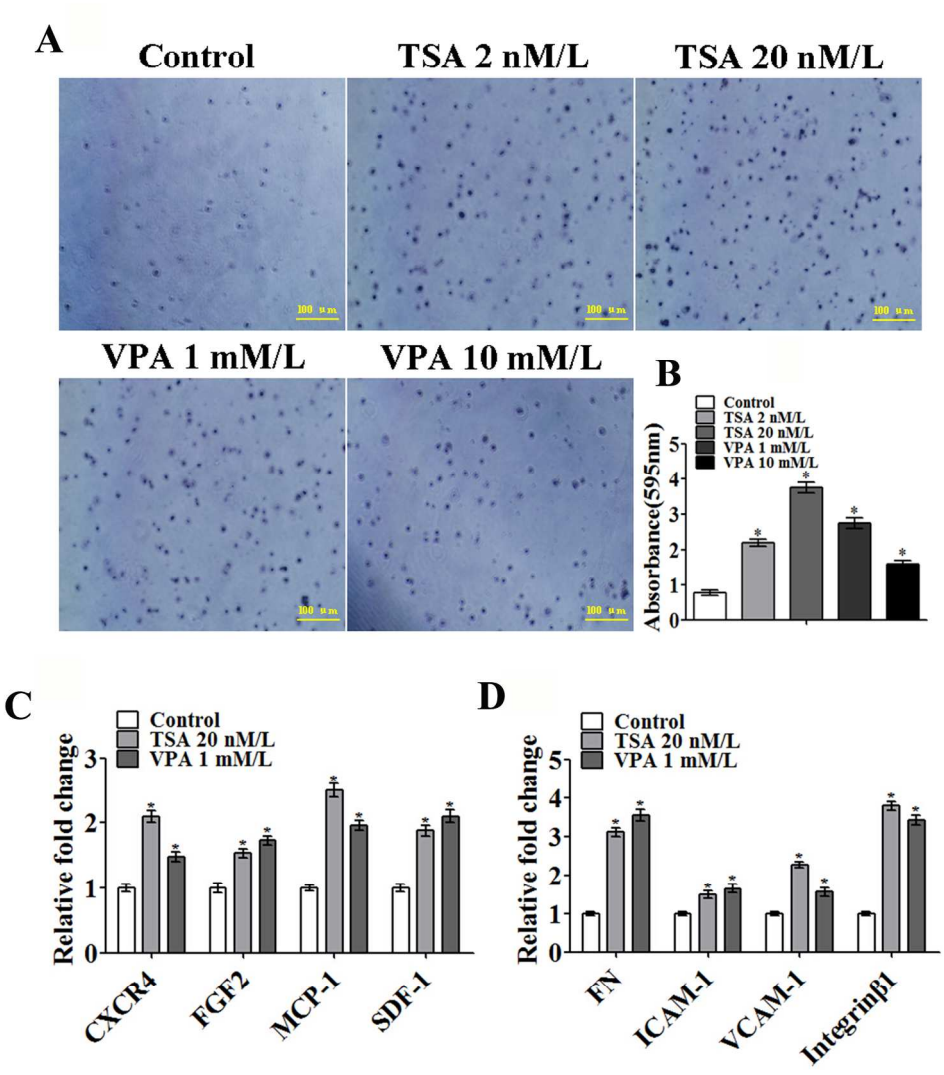
Cell apoptosis- and cycle-related protein expression in hDPSCs, as measured by Western blot analysis (A&B). The levels of chemokines and adhesion molecules were examined by Western blot analysis (C-D). Data are the mean±S.D. of three independent experiments. *P < 0.05 represents a significant change compared with the control.

209x297mm (300 x 300 DPI)



A) Photomicrographs of the scratch were taken at 0 and 24 hours post wounding. C) Quantitative data from A) showing the number of migrated of cells. B) Cell migration assays were applied using a two-chamber Transwell system. D) Quantitative data from B) showing cell migration in response to HDACis. Data are the mean±S.D. of three independent experiments. *P < 0.05 represents a significant difference compared with the control. Scale bars indicate 100 μm.

415x428mm (96 x 96 DPI)



The effects of HDACis on the adhesion of hDPSCs. Cells were incubated in HDACis. A) Adherent cells were fixed and stained and B) quantification was performed. The mRNA expression of chemokines (C) and adhesion(D) molecules examined in hDPSCs. Results are expressed as the mean±standard deviation of 3 independent experiments. *P < 0.05 represents a significant difference compared with the control. Scale bars indicate 100 μm.

209x236mm (300 x 300 DPI)